Volume 194, number 2 FEBS 3241 January 1986

The binding of the B-chain of ricin to Burkitt lymphoma cells

A new approach to ligand-receptor interaction studies

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Received 30 September 1985; revised version received 23 October 1985

It is shown that conformational changes of receptor proteins brought about by binding of a ligand induce changes in the lipid environment of the receptor that can be monitored by fluorescent lipid probes. On this basis a new approach to studies of ligand-receptor binding is proposed. Using the interaction of the ricin B-chain with Burkitt lymphoma cells as an example and fluorescent labelled sphingomyelin as a probe, the ligand-induced changes of fluorescence anisotropy were shown to be concentration-dependent and to permit determination of the binding constant and the number of receptor-binding sites. The method was found to be specific and highly sensitive, allowing detection of the action of one R_B molecule per cell. Scatchard analysis of the binding of ^{125}I-R_B demonstrated the presence on the cell surface of two binding sites with $K_d \sim 10^{-10}$ and $\sim 10^{-8}$ M, respectively. Only the high-affinity sites were detected by the fluorescence technique. Saturation of these sites resulted in maximum inhibition of protein synthesis.

Ricin receptor *Fluorescent probe* *Sphingomyelin* *(Burkitt lymphoma cell)*

1. INTRODUCTION

Receptor-mediated binding of a ligand to the cell surface results in conformational changes of the receptor protein which in turn can induce changes in the molecular organization of the lipid environment of the receptor. Here, we describe an approach to the study of such changes, which is based on the use of lipid-specific fluorescent probes, i.e. modified natural phospholipids bearing a fluorescent label attached to the end of one hydrocarbon chain. It was previously demonstrated that such probes mimic the behavior of natural lipids [1–3] and are able to penetrate into the boundary regions of membrane proteins [2]. Being flexible molecules, the fluorescent labelled lipids in the boundary region will to some extent reflect conformational changes of the receptor protein. Based on these considerations we attempted to assay the binding of the B-chain of ricin to its surface receptor by measuring ricin-induced changes of the steady-state fluorescent anisotropy of the lipid-specific probe ASM incorporated into the plasma membrane of Burkitt lymphoma cells.

The plant toxin ricin consists of 2 polypeptide chains, held together by a disulfide linkage. The function of the A-chain (R_A) is to inhibit protein synthesis in eucaryotic cells, whereas the B-chain (R_B) is responsible for binding of toxin molecules to the cell surface and facilitates penetration of the A-chain into the cell [4]. Since the B-chain has the capacity to bind to any surface glycolipid or glycoprotein containing an accessible terminal

Abbreviations: R_A and R_B, A- and B-chains of ricin; *r*, fluorescence anisotropy; AMS, anthrylvinyl-labelled sphingomyelin, [*N*-12-(9-anthryl)-11-*trans*-dodecanoyl-sphingosine-1-phosphocholine]

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

galactose residue, R_B interacts with a large variety of eucaryotic cells. However the mechanism of interaction of R_B with plasma membranes remains obscure.

2. MATERIALS AND METHODS

2.1. *Cells and fluorescence labelling*

The in vitro-adopted human Burkitt lymphoma cell line EB-3 was obtained from Flow Laboratories. 2 days after subculture EB-3 cells were harvested, washed with buffered saline solution (PBS) and then resuspended to yield a concentration of about 1×10^7 cells/ml. An ethanolic solution of ASM (1 mg/ml) was added to the cell suspension using a microsyringe with intense stirring to a final probe/phospholipid ratio of 1:100 (mol/mol); the ethanol concentration did not exceed 0.5%. Incorporation of the fluorescent probe into cells was controlled by measuring the enhancement of fluorescence intensity, which was maximal after 3 h incubation.

2.2. *Ricin and ricin subunits*

Ricin from *Ricinus communis* seeds was isolated according to [5]. R_A and R_B were obtained as in [6]. The cytotoxicity of ricin was measured by determining the inhibition of [^{14}C]leucine incorporation into Burkitt lymphoma EB-3 cells upon incubation with the toxin. The ID_{50} for ricin was 3×10^{-10} M.

2.3. *Binding of ^{125}I-R_B to cells*

^{125}I-R_B ($1\text{–}0.5 \times 10^6$ cpm/μg) was obtained according to [7], with some modifications. Isolated EB-3 cells were suspended in PBS and an aliquot transferred to a glass tube which had been coated with 0.1% bovine serum albumin in PBS. The cell suspension was then mixed with different amounts of ^{125}I-R_B in 900 μl PBS. After incubation for 10 min at 37°C the cells were washed twice with PBS and then collected and counted.

2.4. *Fluorescent probes and fluorescence measurement*

ASM was synthesized according to [8]. Fluorescence spectra were recorded on a Hitachi 650-60 spectrofluorimeter with a thermostatted cell in quartz cuvettes (5 × 5 mm); the slit width was 2 nm for excitation and 10 nm for emission. Fluorescence anisotropy was calculated by the processor of the spectrofluorimeter.

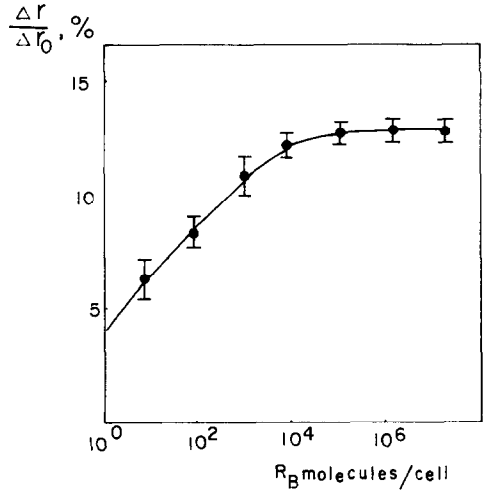

Fig.1. R_B dose-response of the increase in fluorescence anisotropy of ASM-labelled Burkitt lymphoma cells. $\Delta r = r - r_0$, where r_0 and r are fluorescence anisotropy values before and after incubation (37°C, 10 min) of the cells with R_B. Values are averages of triplicate incubations at each concentration from 4 independent experiments.

3. RESULTS AND DISCUSSION

Burkitt lymphoma cells were labelled with ASM and the effect of R_B on the steady-state fluorescence anisotropy of the probe was investigated. A time-course study of the influence of the polypeptide on ASM fluorescence anisotropy demonstrated that the effect is complete within 10 min (not shown). The dose-response relationship of the effect of R_B on ASM labelled EB-3 cells is shown in fig.1. Addition of R_B increased the fluorescence anisotropy in membrane domains sampled by the probe in a concentration-dependent saturable manner. Detectable effects were noted at 10^{-14} M, i.e. at one R_B molecule per cell, and the response was fully saturated at 10^{-9} M (about 10^5 R_B molecules per cell). No further changes in fluorescence anisotropy of the ASM could be detected at higher R_B concentration.

If the ligand-receptor interaction is regarded as a simple reversible bimolecular equilibrium, the association constant K_a can be expressed as

$$K_a = \frac{[RL]}{[L]\,[R]} \qquad (1)$$

where [L] is the concentration of free ligand, [R] that of unoccupied binding sites and [RL] that of ligand-receptor complexes.

We assume that (i) one ligand molecule binds to one receptor molecule and (ii) the change in fluorescence anisotropy of the lipid probe induced by ligand-receptor binding is proportional to the number of occupied binding sites, N.

$$\Delta r = (\Delta r_{max})N \quad (2)$$

where Δr_{max} is the maximal change of r observed when the concentration of the ligand reaches its saturation value. N is defined as

$$N = \frac{[RL]}{[R] + [RL]} \quad (3)$$

Substituting the values of eqn 3 for those of eqn 1 we obtain

$$N = \frac{K_a[L]}{1 + K_a[L]} \quad (4)$$

Combination of eqns 2 and 4 leads to the expression

$$\frac{\Delta r_{max}}{\Delta r} = 1 + K_d \frac{1}{[L]},$$

$$\text{where } K_d = \frac{1}{K_a} \quad (5)$$

If eqn 5 is valid, i.e. if the assumption of proportionality between r and the number of bound ligand molecules is true, the plot of $\Delta r_{max}/\Delta r$ vs $1/[L]$ on double-logarithmic coordinates should have a linear regression. In the case of interaction of Burkitt lymphoma cells with the B-chain of ricin this condition proved to be true (fig.2), consequently in this case eqn 5 may be used for determination of K_d. The K_d value obtained in this way was 5×10^{-11} M. This value represents an upper limit; the true value may be even lower because in our calculation we used the total ligand concentration instead of [L].

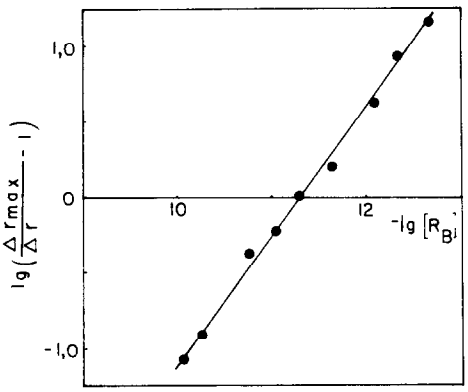

Fig.2. Plot of $\Delta r_{max}/\Delta r$ vs the concentration of R_B on logarithmic coordinates.

The direct proportionality between the probe response and the R_B concentration indicates that those probe molecules which during the incubation eventually may enter the cytoplasm do not influence the ligand-induced changes of r.

In radioligand binding assays of R_B the extent of specific binding may be evaluated by preliminarily adding excess galactose. The amount of R_B which binds to cells under such conditions corresponds to specific interaction. With most cells and some virus particles specific R_B binding comprises 80–95% of the overall binding [4,9,10]. According to radioligand measurements for Burkitt lymphoma cells specific binding of R_B amounts to 85% of the overall binding (not shown). At the same time r is not altered when R_B is added to the cells in the presence of excess galactose. Moreover, the fluorescence anisotropy of the lipid probe is not changed upon addition of R_A which is known to bind in a nonreceptor-mediated fashion [10]. Evidently the changes of r induced by addition of R_B to the cell suspension must be due to specific

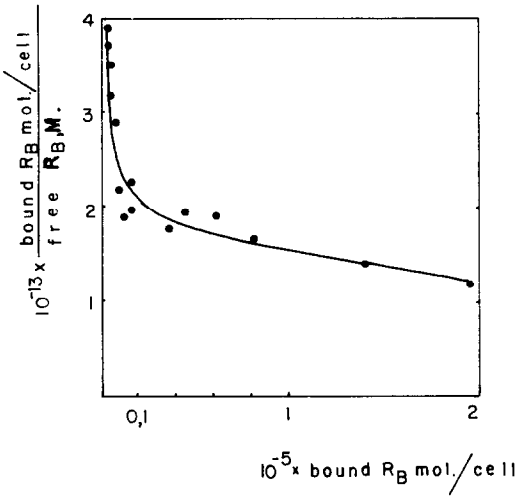

Fig.3. Scatchard plot for the binding of ^{125}I-R_B to Burkitt lymphoma cells. ^{125}I-R_B was added to 10^6 EB-3 cells in 1 ml PBS to a final concentration from 3×10^{-11} to 3×10^{-8} M

binding of the B-chain to cell surface receptors. The nonlinear Scatchard plot (fig.3) suggests the existence of at least 2 binding sites with different affinity. The K_d values derived from Scatchard analysis were $\sim 10^{-10}$ and $\sim 10^{-8}$ M, respectively. The number of high-affinity binding sites obtained by the 2 methods coincided and comprised $\sim 10^4$ per cell.

The results obtained demonstrate that at low R_B concentration the fluorescence method senses only high-affinity binding of the ligand to a certain class of receptor proteins, whereas the radioligand assay measures R_B binding to any membrane component containing a nonreducing terminal galactose residue. This conclusion was supported by the fact that no changes of fluorescence anisotropy were observed when R_B was added to ASM-labelled unilamellar vesicles made from egg yolk phosphatidylcholine-lactosylceramide (5:1). Obviously, the site with higher affinity will show the highest percentage of binding at low ligand concentrations. Provided that the affinity difference is large enough, the 'high-affinity' site will approach saturation before binding to the second site becomes significant.

In this connection it is of interest that the R_B concentration required for maximal increase of r parallels that required for maximal ricin inhibition of [^{14}C]leucine incorporation (not shown). This observation indicates that the saturable R_B effects on r occur at physiologically relevant toxin concentrations.

Comparison of the results obtained by fluorescent and radioligand measurements reveals 2 important advantages of the formed method:

(i) The fluorescence method is much more sensitive than the radioligand binding assay. As can be seen from fig.1 the lipid fluorescent probe permits registering of the action of a single R_B molecule per cell. At such a low concentration the radioligand assay is not able to detect measurable binding.

(ii) At low ligand concentration fluorescence anisotropy changes of the probe molecules appear to reflect only the specific binding.

In principle, ligand-receptor interaction could also be followed by measuring ligand-induced changes in intrinsic protein fluorescence. However, due to the relatively small number of molecules of a given receptor protein in the cell such changes may be difficult to detect. The fluorescence anisotropy changes observed with fluorescent lipid probes are much more pronounced because they result from a cooperative rearrangement of large numbers of lipid molecules.

On the basis of the present results we propose to use ligand-induced fluorescence anisotropy changes in targets labelled with fluorescent analogues of membrane lipids as an additional binding assay. The combination of fluorescent and radioligand methods could open new possibilities in ligand-receptor interaction studies.

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